Synthesis and Structure-activity Relationships of Flavonoids Derived from Scutellaria baicalensis Georgi as Potent Anti-flu Agents against Tamiflu-resistant H1N1 Virus and H3N2 Virus

An-Rong Lee[1], Chia-Wei Liao[1], Wen-Liang Chang[1], Chen-Wen Yao[2] and Wen-Hsin Huang[1*]

[1]School of Pharmacy, National Defense Medical Center, Taiwan

[2]Deparment of Pathology and Parasitology, Tri-Service General Hospital, National Defense Medical Center, 161 Minchuan East Rd. Sec. 6, Taipei, Taiwan

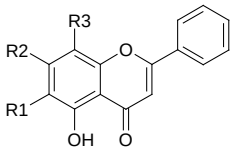
lar@ndmctsgh.edu.tw; littlefire0518@hotmail.com; wlchang@ndmctsgh.edu.tw; yaocw329@gmail.com; wenhsin@ ndmctsgh.edu.tw

***Abstract*-Annual global pandemic and/or epidemic influenza threats continually threaten human beings. Despite annual vaccination and the use of four approved antiviral agents (amantadine, rimantadine, zanamivir, and oseltamivir) to control viral infectivity of influenza viruses, there is still a limited effectiveness and suffering of severe drug side effects as well as the emergence of drug-resistant variants after treatments that prompt the needs of new agents to be developed to treat flu. Some Chinese medicines have been shown potential treatment for influenza. In this study, the direct acylation of dimethoxyphenol with substituted cinnamoyl chlorides by fries reaction and cyclization affords a practical route as standard manipulations for the synthesis of novel flavonoids related to baicalein/oroxyllin-A, which functionalized on the A- and/or B-ring with good overall yields. All the flavonoids in this study were screened with anti-influenza activity against influenza virus (H1N1-Tamiflu resistant and H3N2)-infected in MDCK cell line. Our results showed that most of the synthetic products possessed significant activity. Among them, 4a, 4c, 5c were more potent than that of ribavirin (a positive control) against H1N1-Tamiflu resistant virus. The most potent compound was 4a that displayed an effectively anti-H1N1-Tamiflu resistant viral activity at 2.9 μM and a selectivity index > 103.4. Compound 4c and 5c also exhibited a good inhibitory activity in 5.2 and 5.4 μM, respectively, against H1N1-Tamiflu resistant virus and the corresponding selectivity index > 57.7 and 8.0, respectively. Moreover, in the screening test against H3N2 virus, some synthetic compounds in this study displayed more potent inhibitory activity than against H1N1-Tamiflu resistant virus. Among them, compounds 3a, 5a, 7c, 10 and 11 exhibited a good inhibitory action in 5.5, 6.2, 4.5 4.0 and 6.1 μM against H3N2 virus, and the corresponding selectivity index > 54.4, > 48.3, 4.0, 32.4 and > 49.2, respectively. Those findings in this study provide somewhat important chemical structural features of flavonoids relating their ability after SAR analysis to control the replication of influenza virus and would provide basis to expedite the design and development of chemical compounds with higher potency and lower toxicity to serve as potential NA inhibitors for influenza treatment.**

Keywords-Baicalein; Flavonoids; Anti-influenza Virus; Tamiflu-resistant H1N1 Virus; H3N2 Virus

I. INTRODUCTION

In previous study, we focused on antioxidative, anti-inflammatory, and anti-aggregatory activities of flavonoids, baicalein, baicalin, baicaleinyl sulfate, oroxylin-A, and wogonin, derived from Scutellaria baicalensis GEORGI [1-3]. Currently the extensive efforts are made on a continuous search for new chemo-protective and cyto-protective agents. We have further examined the anti-viral activity of baicalein, baicalin, baicaleinyl sulfate, oroxylin-A, and wogonin (Fig. 1 and Table Ⅰ), and have synthesized novel polyphenolics, structural modification at 6- or 7-position of flavone scaffold, related to baicalein and oroxylin-A, which are naturally occurring constituents of Scutellaria baicalensis Georgi and other forages as potential anti-influenza agents.



	Substituent		
Flavone	R1	R2	R3
Baicalein	OH	OH	H
Baicalin	Glu	OH	H
Baicaleinyl sulfate	OH	OSO_3H	H
Oroxylin-A	OCH_3	OH	H
Wogonin	H	H	OCH_3

Glu: glucurnonide.

Fig. 1 Structures of flavones derived from Scutellaria baicalensis Georgi

Exposure to the highly pathogenic influenza A and/or B virus is responsible for the substantially global pandemic threats. The neuraminidase inhibitors (NAIs) oseltamivir (Tamiflu) and zanamivir (Relenza) are widely used in clinical treatment and prevention of influenza. Neuraminidase (NA), one of the two glycoproteins on the surface of influenza virus, catalyzes the cleavage of sialic acid residues to facilitate infection of the virus in the respiratory tract. Because of the close corresponding conserved residues of the active sites of NAs of all influenza A and B viruses, this enzyme has been regarded as a drug target based on sialic acid transition state for drug development for the treatment of influenza [4-11].

Despite annual vaccination of viral infectivity to provide limited control annually and four antiviral agents, namely amantadine, rimantadine, zanamivir, and oseltamivir, currently

TABLE I IN VITRO ANTI-INFLUENZA ACTIVITIES OF FLAVONOIDS IN MDCK CELLS USING CPE ASSAY

Compound	(H1N1-Tamiflu resistant virus)			H3N2		
	EC_{50} (μM)	CC_{50} (μM)	SI	EC_{50} (μM)	CC_{50} (μM)	SI
Baicalein	46.2	> 300	> 6.5	92.3	> 300	> 3.3
Baicalin	69.3	> 300	> 4.3	103.9	> 300	> 2.9
Baicaleinyl sulfate	213.6	> 300	> 1.4	213.6	> 300	> 1.4
Oroxylin A	43.9	> 300	> 6.8	87.7	> 300	> 3.4
Wogonin	43.9	> 300	> 6.8	87.7	> 300	> 3.4
3a	44.3	> 300	> 6.8	5.5	> 300	> 54.5
3b	82.6	> 300	> 3.6	39.0	> 300	> 7.7
3c	40.9	> 300	> 7.3	19.3	> 300	> 15.5
4a	< 2.9	> 300	> 103.4	----	186.4	ND
4b	41.3	> 300	> 7.3	82.6	> 300	> 3.6
4c	5.2	> 300	> 57.7	----	> 300	ND
5a	49.2	> 300	> 6.1	6.2	> 300	> 48.3
5b	55.1	> 300	> 5.4	43.9	> 300	> 6.8
5c	5.4	43.3	8.0	21.7	43.3	2.0
6a	35.2	> 300	> 8.5	37.2	> 300	> 8.1
6b	67.6	> 300	> 4.4	71.4	> 300	> 4.2
6c	33.7	> 300	> 8.9	67.4	> 300	> 4.5
7a	----	40.3	ND	----	20.1	ND
7b	----	74.4	ND	----	30.6	ND
7c	----	9.1	ND	4.5	18.1	4.0
9	ND	ND	ND	ND	ND	ND
10	32.3	129.4	4.0	4.0	129.4	32.4
11	97.6	> 300	> 3.1	6.1	> 300	> 49.2
12	< 9.3	37.2	> 4	----	37.2	ND
13	36.5	> 300	> 8.2	----	219.1	ND
Ribavirin	6.4	> 300	> 46.8	12.8	> 300	> 23.4

EC_{50}: mean value the 50% effective concentration; CC_{50}: mean value the 50% cell cytotoxic concentration; SI: selective index, CC_{50}/EC_{50}; ----: compound synergized cell-infected cell cytotoxicity (cell viability below 50%, CC_{50}); ND: not determined.

it is approved by the FDA to treat influenza virus infection, suffering of several drug toxic effects as well as the emergence of drug-resistant variants of drug treatments impacts to develop new agents to treat viral disease. In principle, to effectively inhibit viral adsorption to epithelial cells, viral intrusion into cells, transcription and replication of viral genomes, viral protein expression, and progeny virus release from cells are the significantly distinct targets for evoking to develop novel antiviral agents [7, 8]. Among them, in particular the current available drugs for treating influenza virus infection, there are M2 ion channel blockers, amantadine, rimantadine, and two major neuraminidase inhibitors (NAIs), namely oseltamivir (Tamiflu) and zanamivir (Relenza) that are extensively used in medical treatment and/or prevention of influenza.

Even though they have high efficacy of antiviral activity of both oseltamivir and zanamivir, unfortunately, its low bioavailability and rapid nephrotic excretion of zanamivir, and frequent nausea, vomiting, diarrhea, abdominal pain, and headache associated with oseltamivir therapy and high dangerous neuropsychiatric side effects even including self-harm after receiving oseltamivir are still need to be considered [12]. Moreover, recent studies have proven that oseltamivir-resistant pandemic H1N1 viral isolates have been progressively rising [13-15]. The threat of annual pandemics requires the development of new therapeutic agents.

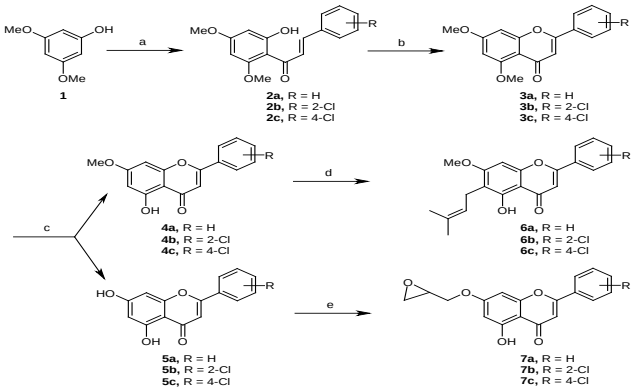
Apart from antiviral agents, plant phenolics, an important class of naturally derived phytomedicines of interests against influenza flu, avian flu and other diseases, reveal the true potential prevention and/or treatment for flu. Currently several flavonoids derived from plants such as kaempferol (IC_{50} = 30.2μM), isoscutellarein (IC_{50} = 20 μM) isolated from Rhodiola rosea and Scutellaria baicalensis, respectively, have been

reported that they had anti-influenza virus activity by inhibiting NA activity [16-23].

In this study, the ability of the synthetic flavonoids based on the standard manipulation of preparing baicalein and oroxylin-A to interfere with the plaque formation by human influenza viruses was examined on the basis of the maximum non-toxic concentration of the test compounds. Some of our products showed the most potent activity lower to 2.9 and 4.0 μM, in vitro, against H1N1-Tamiflu resistant virus and seasonal H3N2 virus, respectively. Further investigations on the mechanisms of action are in progress.

II. RESULTS

Two influenza virus strains, oseltamivir (Tamiflu)-resistant, the year 2009 pandemic influenza A (H1N1) virus, which detected the H275Y mutation (N1 numbering) in neuraminidase and Influenza A/New York/469/2004-like flu virus, were provided by Centers for Disease Control (CDC, Taiwan) and adapted for evaluating flavonoids of in vitro anti-viral activities using a cytopathic effect (CPE) reduction assay in virus-infected MDCK cell line. Those findings of this study provide important structure activity relationships for the exploitation and utilization of flavonoids as potential NA inhibitors for influenza treatment. In this study, the synthetic flavonoids were categorized into different subgroups and investigated for their inhibitory effect, and their synthesized approaches and molecular structures are shown in Fig. 2. Their compound and screening data are listed in Table Ⅰ according to their subgroup classification.



Reagents
(a) substituted cinnamoyl chloride, BF_3-Et_2O; (b) I_2/DMSO; (c) 47% HBr/ HOAc; (d) KOH, MeOH, 3,3-dimethylallyl bromide, rt; (c) KOH, MeOH, epichlorohydrin.

Fig. 2 Scheme of synthesized flavones

A. Chemistry

In our laboratories, various derived flavonoids related to baicalein and/or oroxylin-A could be readily obtained by structural modification of the standard manipulations. Reaction of 3, 5-dimethoxyphenol with the respective equimolar cinnamoyl chloride provided the corresponding chalcone intermediate quantitatively. Successively intramolecular oxidative cyclization of the respective chalcone with I_2/DMSO, followed by demethylation dependent upon the controlled conditions afforded various functionalized flavonoids (Series 3, 4 and 5, Fig. 2) in excellent yields after purification by column chromatography.

The extended syntheses were *C*-alkylation with 3, 3-dimethylallyl bromide and KOH in methanol derived from compound Series 4 afford compound Series 6, and moreover, *O*-alkylation of compound Series 5 were with epichlorohydrin and KOH in MeOH to make compound Series **7** in moderate yields.

B. In Vitro Anti-influenza Virus Activity

The examined activity of flavonoids in this study was expressed as 50% inhibitory concentration (IC_{50}) against influenza viruses, and CC_{50} meant the 50% cell cytotoxic concentration after compound treatment, wherein selective index was expressed as SI, CC_{50}/EC_{50} and employed in the SAR analysis.

We have further developed a series of flavonoids relevant to baicalein and tested their inhibitory activity against influenza virus (H1N1-Tamiflu resistant and H3N2)-infected in MDCK cell line shown in Table Ⅰ. Our results showed that most of the synthetic products possessed significant activity. Among them, 4a, 4c, 5c were more potent than that of ribavirin (a broad spectrum of antiviral agent as a positive control) against H1N1-Tamiflu resistant virus. The most potent compound was 4a that displayed an effectively anti-H1N1-Tamiflu resistant viral activity at 2.9 μM and a selectivity index > 103.4. Compound 4c and 5c also exhibited a good inhibitory activity in 5.2 and 5.4 μM, respectively, against H1N1-Tamiflu resistant virus and the corresponding selectivity index > 57.7 and 8.0, respectively. Moreover, in the screening test against H3N2 virus, more synthetic compounds in this study displayed more potent inhibitory activity than against H1N1-Tamiflu resistant virus. Among them, compounds 3a, 5a, 7c, 10 and 11 exhibited a good inhibitory action in 5.5, 6.2, 4.5 4.0 and 6.1 μM against H3N2 virus, and the corresponding selectivity index > 54.4, > 48.3, 4.0, 32.4 and > 49.2, respectively.

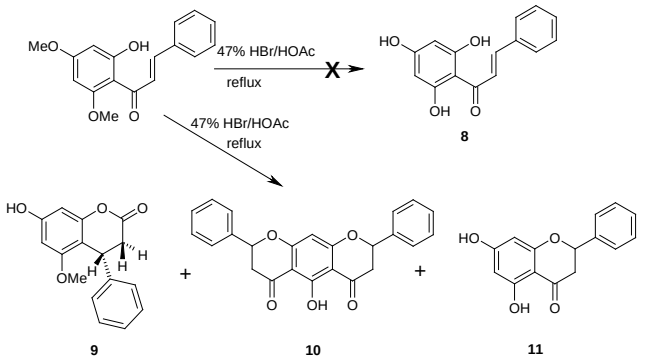
III. DISCUSSIONS

A. Chemistry

In brief, our standard manipulations of synthesized flavones in this study, Fries reaction of 3, 5-dimethoxyphenol with the respective equimolar cinnamoyl chloride afforded the corresponding chalcone intermediate and successively intramolecular oxidative cyclization of the respective chalcone with I_2/DMSO was conducted. The demethylation of substituted flavones dependent upon the controlled conditions yielded various functionalized flavonoids such as series 3, 4 and 5, (Fig. 2) in excellent yields after purification.

In the alternative preparation of compound 5a from 2a through the refluxing condition of 47% HBr/HOAc (1/1) to try to get chalcone 8 for confirming the existence, and followed by sequential cyclization by I_2/DMSO but futile, interestly unexpected side products flavonoid 9, 10, and 11 were obtained in lower as 9.6%, 5.7% and 13.2% yields, respectively, in refluxing 13 hr and in 15.3%, 16.6% and neat, respectively in refluxing 48 hr. (Fig. 3) Encountering this difficulty of making 5a from 2a, the synthetic approaches were back to perform the standard manipulations as Fig. 2 is shown; however, the side product 9 was structural identification and characterization by ir, nmr, and ms spectra, and even crystal x-ray diffraction as Fig. 4 shown as a dihydrocoumarin derived from rearrangement of chalcone 2.

The preparation of Series 6 (*C*-alkylation) from Series 4 and preparation of Series 7 (*O*-alkylation) from Series 5 in KOH/MeOH were successful as expected, whereas acetone instead of MeOH as solvent, the unexpected 5-*O*-dimethylallyloxy-7-methoxylflavone (12) from 2a (Fig. 5)



rxn. time	9 (yield)	10 (yield)	11 (yield)
13 hrs	9.6%	5.7%	13.2%
2 days	15.3%	16.6%	Not exist

Fig. 3 The side products in the alternative preparation of flavones

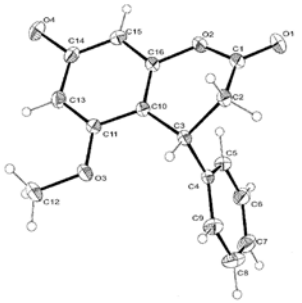


Fig. 4 The x-ray structure of dihydrocoumarin 9

were afforded and 5-hydroxy-7-(3-methylglycidyloxy)-flavone (13) were obtained after purification by column chromatography as well.

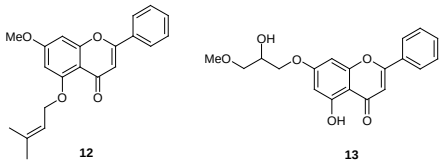


Fig. 5 The structures of flavone 12 and 13

B. In Vitro Anti-influenza Virus Activity

Even though flavonoids are a kind of well-known C6-C3-C6 phenylbenzopyrone scaffold, few reports have been focused on the anti-influenza virus activities of several flavonoids derived from plants or Chinse medicines in the past [16-23]. The experimental data is not enough to fully elucidate mechanisms for their action and intact SARs due to their structural diversity resulted in the difficulties of the SAR analysis. In this study, we focused on flavone compounds derived from Scutellaria baicalensis Georgi to elucidate the somehow structural features of flavonoids in vitro anti-influenza viral effect and to provide new experimental data for the future exploitation and utilization of flavonoids as potential anti-influenza medicines.

First of all, baicalein, baicalin, baicaleinyl sulfate, oroxylin-A, and wogonin displayed weaker in vitro anti-influenza virus activities than that of ribavirin shown in Table Ⅰ. Baicalein, oroxylin-A, and wogonin exhibited the comparable moderate activity. Baicalin and baicaleinyl sulfate had dramatically reduced inhibition compared to baicalein, hence it seems that the decrease of the anti-viral effects was due to steric hindrance and higher polarity in the presence of the bulky 6-glycosylation or 7-sulfate conjugation.

A representative coplanar A,C-ring of flavone 5a exhibited an effective level against H1N1-Tamiflu resistant virus H3N2 virus at 49.2, 6.2 μM, respectively and shown non-cytotoxic effect (> 300 μM), SI > 6.1 and > 48.3, respectively.

The synthesized flavanone 11, a non-coplanar flavonoid, had less anti- H1N1-Tamiflu resistant virus activity (97.6 μM) and non-cytotoxic effect, but it extraordinarily held a strong activity against H3N2 virus at level 4.0 μM and SI = 32.4. Another flavanone 10, 8-phenyl-7,8-dihydropyranoflavanone, exhibited both anti-viral activity against H1N1-Tamiflu resistant virus and H3N2 virus at level 32.3, and 6.1 μM, respectively and cytotoxic effects at 129.4 μM. This seems that the enzymes of H3N2 virus have much broad and deep cavity suitable for flavanone docking.

The comparison among compounds 3a, 4a, 4b, 4c and 12, bearing the corresponding 7-OCH_3 at A-ring, indicated that the inhibitory activity of those compounds against H1N1-Tamiflu resistant virus obviously exhibited moderate to good activity but inconspicuous activity against H3N2 virus due to synergistic cytotoxicity. Among them, the most potent compound is 4a, a lack of methyl moiety at 5-position of A-ring exhibiting an intra-molecular H-bonding, which held more potent and toxic than that of 3a against H1N1-Tamiflu resistant virus and H3N2 virus, respectively. Compound 12, a 5-*O*-prenylation of 4a, displayed a slight decrease activity against H1N1-Tamiflu resistant virus and toxic for H3N2 virus as well.

The comparison of compounds 4b, 4c, 5b, 5c, 6b, 6c, 7b, and 7c, bearing a 2'-Cl or 4'-Cl functionality at the B-ring of flavones, displayed that apparently reduced the activity to 4a, 5a, 6a, and 7a (no substituent at the B-ring), while c series (with 4'-Cl substituent) still held the comparable activity, but b series (with 2'-Cl substituent) dramatically decrease the effect that might be a 2'-Cl substituent resulting in steric hindrance and loss of C6-C3-C6 concurrent coplanar force.

The comparison of 6a, 6b, 6c, the corresponding 6-*C*-prenylation of 4a, 4b, 4c, respectively, showed that bulky prenyl moiety disfavored the effect. On the other hand, a 5-*O*-prenylated compound 12 derived from 4a in acetone exhibited more potent and toxic against both H1N1-Tamiflu resistant virus and H3N2 virus than that of 6a. In general, 7a, 7b, 7c, the corresponding 7-*O*-glycidylation of 5a, 5b, 5c, respectively, indicated that exhibited more toxic against both H1N1-Tamiflu resistant virus- and H3N2 virus-infected MDCK cells resultant in inconspicuous inhibitory effects on virus, whereas only 7c held an IC_{50} = 4.5 μM at a selectivity index = 4.

The overall comparisons of flavonoids are shown in Table Ⅰ. The order of potency for NA inhibition seems as follows: flavones > flavanones. For good inhibitory activity of flavonoids for viruses, it seems that flavonoids with a flavone skeleton bearing C2=C3, and C4=O functionalities are essential, and bearing 5- and/or 7-*O*-substituents are important. Halo-substituent such as Cl at B-ring of flavones might have stronger or comparable anti-influenza activity especially at 4'-Cl but not at 2'-Cl.

Ⅳ. MATERIALS AND METHODS

A. Chemicals and Reagents

Ribavirin and baicalin hydrate with 98% purity, Fetal Bovine Serum (FBS), dimethyl sulfoxide (DMSO), 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), trypan blue, and Dulbecco's minimum essential medium (DMEM) were purchased from Aldrich-Sigma Chemical Company (St. Louis, MO, USA). All the other chemicals, unless otherwise specified, for the use of synthesis were purchased from Aldrich-Sigma Chemical Company (St. Louis, MO, USA) or Alfa-Aesar Chemical Company (Heysham, LA32XY, England). Trypsin–EDTA and trypsin (1:250) were purchased from the Gibco Company.

B. Chemical Synthesis and Characterization

Melting points were taken in open capillary tubes on a Buchi-530 melting point apparatus and were uncorrected. UV-VIS spectra were recorded on a Shimazu UV-160A UV-Visble recording spectrophotometer. IR spectra were recorded on a Perkin-Elmer FTIR 1610 series infrared spectrophotometer in KBr discs or CH_2Cl_2 solution. ^{1}H- and ^{13}C-NMR spectra were determined on a Varian Gemini-300 NMR instrument. Chemical shifts (δ) were reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard, and coupling constants (*J*) were given in hertz (Hz). Electronic ionization (EI) and electro-spray ionization (ESI) mass spectra were recorded using a Micromass TRIO-2000 (GC/MS) and liquid chromatography tandem (Q-Tof LC/MS/MS) mass spectrometer. All reactions were routinely monitored by TLC on Merck F254 silica gel plates. Merck silica gel (70-230 mesh) was used for chromatography. High resolution mass spectra were performed in the Instrument Center of the National Science Counsel at the National Tsinghua University using Finnigan MAT-95XL. All the solvents and reagents were obtained from commercial sources and purified before use if necessary.

The flavonoids, baicalein, baicaleinyl sulfate, oroxylin-A, and wogonin were synthesized as described previously [1-3]. Other novel flavonoids in this study were synthesized as described as follows:

1) General Procedures for Preparation of Substituted Cinnamoyl Chlorides:

A solution of substituted cinnamic acid (10 mmol) in dichloromethane (25 ml) at 0 ℃ was added oxalyl chloride (1 ml, 11 mmol) and *N*, *N*-dimethylformamide (2 drops). The reaction mixture was stirred at 0 ℃ for 5 minutes and then allowed to be stirred at room temperature for 1 h, and then concentrated under reduced pressure to dryness. The mixture was used for further synthesis without purification.

2) General Procedures for the Synthesis of (E)-1-(2-hydroxy 4,6-dimethoxyphenyl)-3-(substituted phenyl)-prop-2-en-1-ones (2):

The equimolar of respective substituted cinnamoyl chloride (10 mmol, R = H, 2-Cl, or 4-Cl) and 3, 5-dimethoxyphenol (13, 1.5 g, 10 mmol) was dissolved in BF_3-Et_2O complex (5 mL), respectively. The mixture was heated at reflux for 5 minutes. After cooling, the mixture was poured with excess of ice water (100 ml) to precipitate the product. After filtration, the filtrate was washed with water until free from acid and then purified by column chromatography (hexane: EtOAc=3:1) to get the corresponding chalcone 2a (2.6 g, 9.1 mmol, yield: 89%), 2b (2.7 g, 8.5 mmol, yield: 85%) or 2c (2.7 g, 8.5 mmol, yield: 85%), respectively, as orange-yellow powders.

3) General Procedures for the Synthesis of 5,7-dimethoxy-2-(substituted phenyl)-4H-chromen-4-ones (3):

10 mmol of the respective chalcone 2a ~ 2c (R = H, 2-Cl, or 4-Cl) and iodine (25 mg) in DMSO (20 mL) was refluxed for 3 hours, respectively. After cooling, the mixture was poured into ice-water (100 ml) and then treated with saturated $Na_2S_2O_3$ solution (100 mL) to precipitate the product. After filtration, the filtrate was washed with water until free from DMSO and recrystallization from dichloromethane to get the corresponding pure 3a (2.3 g, 8.1 mmol, yield: 81%), 3b (2.4 g, 7.6 mmol, yield: 76%) or 3c (2.3 g, 7.2 mmol, yield: 72%), respectively, as colorless crystals.

4) General Procedures for the synthesis of 5-hydroxy-7-methoxy-2-(substituted phenyl)-4H-chromen-4-ones (4) and 5,7-dihydroxy-2-(substituted phenyl)-4H-chromen-4-ones (5):

5 mmol of the respective flavone 3a ~ 3c (R = H, 2-Cl, or 4-Cl) was dissolved in 48% HBr (10 mL) and glacial acetic acid (10 mL), and then refluxed for 15 hours. After cooling, the reaction mixture was poured into ice-water to precipitate the crude product. After filtration, a yellow powders was washed with water until free from acid and then purified by column chromatography (hexane: EtOAc=2:1) to get the corresponding pure 4a (575 mg, 2.15 mmol, yield: 43%), 4b (530 mg, 1.75 mmol, yield: 35%) or 4c (490 mg, 1.63 mmol, yield: 33%), respectively, and the corresponding pure 5a (515 mg, 2.04 mmol, yield: 41%), 5b (420 mg, 1.45 mmol, yield: 29%) or 5c (300 mg, 1.04 mmol, yield: 21%), respectively. All compounds 4 and 5 are yellow powders.

5) General Procedures for the synthesis of 5-hydroxy-7-methoxy-6-(3-methylbut-2-en-1-yl)-2-(substituted phenyl)-4H-chromen-4-ones (6):

2 mmol of the respective flavone 4a ~ 4c (R = H, 2-Cl, or 4-Cl), 1-bromo-3-methylbut-2-ene (1.1 ml, 10 mmol), and KOH (0.55 g, 10 mmol) in methanol (30 mL) was stirred at room temperature for 1.5 days. The reaction mixture was evaporated *in vacuo*, and the resulting residue was neutralized with saturated $NaHCO_3$ solution to pH 8~9. The aqueous layer was extracted with dichloromethane. The crude product was purified by column chromatography (hexane: EtOAc=3:1) to get the corresponding pure 6a (339 mg, 0.95 mmol, yield: 49%), 6b (287 mg, 0.77 mmol, yield: 39%) or 6c (400 mg, 1.08 mmol, yield: 54%), respectively, as bright yellow powders.

6) General Procedures for the synthesis of 5-hydroxy-7-(oxiran-2-ylmethoxy)-2-(substituted phenyl)-4H-chromen-4-ones (7):

2 mmol of the respective flavone 5a ~ 5c (R = H, 2-Cl, or 4-Cl), epichlorohydrin (1.3 mL, 10 mmol), and KOH (0.55 g, 10 mmol) in methanol (30 mL) was refluxed for 20 hours. After cooling, the reaction mixture was evaporated *in vacuo*, and the resulting residue was neutralized with saturated $NaHCO_3$ solution to pH 8~9. The aqueous layer was extracted with ethyl acetate. The crude product was purified by column chromatography (hexane: EtOAc=3:1) to get the corresponding pure 7a (282 mg, 0.91 mmol, yield: 46%), 7b (230 mg, 0.67 mmol, yield: 33%) or 7c (164 mg, 0.48 mmol, yield: 24%), respectively, as pale yellow powders.

7) 5,7-Dimethoxy-2-phenyl-4H-chromen-4-one (3a):

Rf: 0.59 (EtOAc:Hexane=1:3); mp : 150-151 ℃; UV (MeOH): λ_{max} nm (logε) = 304 (3.57), 267 (3.87), 212 (3.74);

IR (KBr) cm^{-1}: 3092, 3012 (C-H), 1655 (C=O), 1615 (C=C) ; ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.83 (3H, s), 3.90 (3H, s), 6.51 (d, *J* = 2.1 Hz, 1H, H-6), 6.76 (s, 1H, H-3), 6.86 (d, *J* = 2.1 Hz, 1H, H-8), 7.55-7.57 (m, 3H, H-3', 4', 5'), 8.02-8.05 (m, 2H, H-2', 6'); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) = 54.7, 56.2, 93.1, 98.7, 103.6, 105.4, 126.9, 128.1, 129.1, 130.3, 159.1, 162.9, 164.1, 168.1, 182.2; HRMS (EI) *m/z*: calcd for $[M]^+$, 282.0887 ($C_{17}H_{14}O_4^+$), found: 282.0889.

8) 2-(2'-Chlorophenyl)-5,7-dimethoxy-4H-chromen-4-one (3b):

Rf: 0.57 (EtOAc:Hexane=1:3); mp: 167-168 ℃ ; UV (MeOH): λ_{max} nm (logε) = 309 (3.78), 260 (3.92), 220 (3.83); IR (KBr) cm^{-1} : 3092, 2955 (C-H), 1668 (C=O), 1620 (C=C); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 3.85 (s, 3H, -ArOC<u>H</u>$_3$), 3.95 (s, 3H, -ArOC<u>H</u>$_3$), 6.41 (d, *J* = 2.4 Hz, 1H, H-6), 6.57 (s, 1H, H-3), 6.89 (d, *J* = 2.1 Hz, 1H, H-8), 7.45-7.67 (m, 4H, H-2', 3', 5', 6'), 12.65 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz, $CDCl_3$-d_1): δ (ppm) = 55.7, 58.1, 93.2, 98.1, 105.1, 111.3, 126.7, 130.2, 130.6, 131.7, 132.6, 160.3, 162.3, 162.9, 165.1, 182.3; HRMS (EI) *m/z*: calcd for $[M]^+$, 316.1679 ($C_{16}H_{11}ClO_4^+$), found: 316.1675.

9) 2-(4'-Chlorophenyl)-5,7-dimethoxy-4H-chromen-4-one (3c):

Rf: 0.61 (EtOAc:Hexane=1:3); mp: 209-210 ℃ ; UV (MeOH): λ_{max} nm (logε) = 310 (3.89), 274 (3.93), 223 (3.81); IR (KBr) cm^{-1}: 3095, 2980 (C-H), 1674 (C=O), 1621 (C=C); ^{1}H-NMR (300 MHz , DMSO-d_6): δ (ppm) = 3.89 (s, 3H, -ArOC<u>H</u>$_3$), 3.95 (s, 3H, -ArOC<u>H</u>$_3$), 6.51 (d, *J* = 2.4 Hz, 1H, H-6), 6.93 (d, *J* = 2.1Hz, 1H, H-8), 7.05 (s, 1H, H-3), 7.62 (d, *J* = 8.7 Hz, 2H, H-2', 6'), 8.10 (d, *J* = 8.7 Hz, 2H, H-3', 5'), 12.71 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz , $CDCl_3$-d_1): δ (ppm) = 55.9, 58.1, 92.5, 98.3, 106.1, 106.6, 127.3, 129.1, 129.7, 137.9, 157.8, 162.3, 162.7, 166.3, 182.1; HRMS (EI) *m/z*: calcd for $[M]^+$, 316.1679 ($C_{16}H_{11}ClO_4^+$), found: 316.1680.

10) 5-Hydroxy-7-methoxy-2-phenyl-4H-chromen-4-one (4a):

Rf: 0.45 (EtOAc:Hexane=1:3); mp : 170-171 ℃; UV (MeOH) : λ_{max} nm (logε) = 304 (3.68), 266 (3.94), 212 (3.88); IR (KBr) cm^{-1}: 3066 (C-H), 1666 (C=O), 1608 (C=C), 1201 (C-C), 1157 (C-O); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.87 (s, 3H, -ArOC<u>H</u>$_3$), 6.39 (d, *J* = 2.1 Hz, 1H, H-6), 6.80 (d, *J* = 2.1 Hz, 1H, H-8), 7.02 (s, 1H, H-3), 7.57-7.62 (m, 3H, H-3', 4', 5'), 8.07-8.11 (m, 2H, H-2', 6'), 12.80 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz, $CDCl_3$-d_1): δ (ppm) = 56.0, 93.0, 98.5, 106.1, 106.3, 126.6, 129.4, 131.9, 132.1, 158.3, 162.8, 164.4, 166.1, 182.9; HRMS (EI) *m/z*: calcd for $[M]^+$, 268.0736 ($C_{16}H_{12}O_4^+$), found: 268.0737.

11) 2-(2-Chlorophenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (4b):

Rf: 0.47 (EtOAc:Hexane=1:3); mp: 187-188 ℃; UV (MeOH): λ_{max} nm (logε) = 259 (3.94), 216 (3.93); IR (KBr) cm^{-1} : 3066, 2931 (C-H), 1664 (C=O), 1622 (C=C), 1195 (C-C), 1166 (C-O), 1035 (C-Cl); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 3.87 (s, 3H, -ArOC<u>H</u>$_3$), 6.39 (d, *J* = 2.4 Hz, 1H, H-6), 6.83 (d, *J* = 2.1 Hz, 1H, H-8), 6.53 (s, 1H, H-3), 7.41-7.63 (m, 4H, H-2', 3', 5', 6'), 12.63 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz, $CDCl_3$-d_1): δ (ppm) = 55.2, 92.2, 97.8, 105.3, 110.9, 126.6, 130.1, 130.4, 131.4, 132.5, 157.8, 162.0, 162.6, 165.4, 181.7; HRMS (EI) *m/z*: calcd for $[M]^+$, 302.0346 ($C_{16}H_{11}ClO_4^+$), found: 302.0342.

12) 2-(4-Chlorophenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (4c):

Rf: 0.49 (EtOAc:Hexane=1:3); mp: 229-230 ℃; UV (MeOH): λ_{max} nm (logε) = 271 (3.83), 213 (3.91); IR (KBr) cm^{-1}: 2920 (C-H), 1664 (C=O), 1616 (C=C), 1217 (C-C), 1164 (C-O), 1091 (C-Cl); ^{1}H-NMR (300 MHz , DMSO-d_6): δ (ppm) = 3.87 (s, 3H, -ArOC<u>H</u>$_3$), 6.41 (d, *J* = 2.4 Hz, 1H, H-6), 6.83 (d, *J* = 2.1Hz, 1H, H-8), 7.10 (s, 1H, H-3), 7.65 (d, *J* = 8.7 Hz, 2H, H-2', 6'), 8.12 (d, *J* = 8.7 Hz, 2H, H-3', 5'), 12.76 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz , $CDCl_3$-d_1): δ (ppm) = 55.1, 92.2, 97.8, 105.2, 105.6, 127.0, 128.9, 129.5, 137.7, 157.3, 162.0, 162.3, 166.0, 181.8; HRMS (EI) *m/z*: calcd for $[M]^+$, 302.0346 ($C_{16}H_{11}ClO_4^+$). We found: 302.0347.

13) 5,7-Dihydroxy-2-phenyl-4H-chromen-4-one (5a):

Rf: 0.39 (EtOAc:Hexane=1:2); mp: 284-285 ℃ ; UV (MeOH): λ_{max} nm (logε) = 267 (3.87), 212 (3.84); IR (KBr) cm^{-1}: 3600~3200 (broad, O-H), 3012, 2893 (C-H), 1652 (C=O), 1612 (C=C) ; ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 6.21 (d, *J* = 2.1 Hz, 1H, H-6), 6.51 (d, *J* = 2.1 Hz, 1H, H-8), 6.96 (s, 1H, H-3), 7.54-7.61 (m, 3H, H-3', 4', 5'), 8.04-8.07 (m, 2H, H-2', 6'), 10.95 (s, 1H, -C7-O<u>H</u>), 12.82 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) = 94.0, 99.0, 104.0, 105.2, 126.3, 129.0, 130.8, 131.9, 157.5, 161.5, 163.3, 164.5, 181.8; HRMS (EI) *m/z*: calcd for $[M]^+$, 254.0579 ($C_{15}H_{10}O_4^+$); found: 254.0584.

14) 2-(2-Chlorophenyl)-5,7-dihydroxy-4H-chromen-4-one (5b):

Rf: 0.41 (EtOAc:Hexane=1:2); mp: 274-275 ℃ ; UV (MeOH): λ_{max} nm (logε) = 263 (3.87), 212 (3.84); IR (KBr) cm^{-1}: 3600~3200 (broad, O-H), 2906 (C-H), 1652 (C=O), 1612 (C=C), 1170 (C-O), 1028 (C-Cl); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 6.24 (d, *J* = 1.8 Hz, 1H, H-6), 6.40 (d, *J* = 2.1 Hz, 1H, H-8), 6.60 (s, 1H, H-3), 7.53-7.78 (m, 4H, H-2', 3', 5', 6'), 10.98 (s, 1H, -C7-O<u>H</u>), 12.66 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) = 94.0, 99.0, 103.8, 110.5, 127.6, 130.4, 130.9, 131.2, 131.6, 132.6, 157.8, 161.6, 162.7, 164.7, 181.4; HRMS (EI) *m/z*: calcd for $[M]^+$, 288.0189 ($C_{15}H_9ClO_4^+$), found: 288.0193.

15) 2-(4-Chlorophenyl)-5,7-dihydroxy-4H-chromen-4-one (5c):

Rf: 0.43 (EtOAc: Hexane=1:2); mp: 270-271 ℃ ; UV (MeOH) : λ_{max} nm (logε) = 271 (3.99), 214 (4.01); IR (KBr) cm^{-1}: 3095, 2916 (C-H), 1654 (C=O), 1618 (C=C), 1166 (C-O), 1096 (C-Cl); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 6.21 (d, *J*=1.8 Hz, 1H, H-6), 6.51 (d, *J*=1.8 Hz, 1H, H-8), 7.01 (s, 1H, H-3), 7.62 (d, *J*=8.4 Hz, 2H, H-2', 6'), 8.08 (d, *J*=8.7 Hz, 2H, H-3', 5'), 10.96 (s, 1H, -C7-O<u>H</u>), 12.77 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz, acetone- d_6): δ (ppm) = 95.3, 100.4, 106.0, 107.0, 129.3, 130.5, 131.6, 138.7, 159.4, 163.9, 164.0, 165.7,

183.5; HRMS (EI) *m/z*: calcd for $[M]^+$, 288.0189 ($C_{15}H_9ClO_4^+$); found: 288.0191 *m/z*.

16) 5-Hydroxy-7-methoxy-6-(3-methylbut-2-en-1-yl)-2-phenyl-4H-chromen-4-one (6a):

Rf: 0.48 (EtOAc:Hexane=1:3); mp: 184-185 ℃; UV (MeOH): λ_{max} nm (logε) = 269 (4.08), 214 (4.01); IR (KBr) cm^{-1}: 2906 (C-H), 1658 (C=O), 1608, 1585 (C=C), 1205 (C-C), 1166 (C-O); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 1.69 (s, 3H, -CHC($C\underline{H}_3$)$_2$), 1.81 (s, 3H, -CHC($C\underline{H}_3$)$_2$), 3.53 (s, 1H, 1 of C6-C$\underline{H}_2$), 3.55 (s, 1H, 1 of C6-C$\underline{H}_2$), 5.22 (t, *J*=1.8 Hz, 1H, -C$\underline{H}$C(CH_3)$_2$), 6.42 (s, 1H, H-8), 6.66 (s, 1H, H-3), 7.50-7.56 (m, 3H, H-3', 4', 5'), 7.89-7.92 (m, 2H, H-2', 6'), 12.81 (s, 1H, -C5-O$\underline{H}$); ^{13}C-NMR (75 MHz, $CDCl_3$-d_1): δ (ppm) = 17.2, 21.1, 24.9, 55.4, 94.8, 104.8, 104.9, 107.7, 121.9, 125.8, 128.5, 131.1, 131.3, 131.4, 154.1, 160.3, 162.6, 163.5, 182.5; HRMS (EI) *m/z*: calcd for $[M]^+$, 336.1362 ($C_{21}H_{20}O_4^+$), found：336.1357.

17) 2-(2-Chlorophenyl)-5-hydroxy-7-methoxy-6-(3-methylbut-2-en-1-yl)-4H-chromen-4-one (6b):

Rf: 0.50 (EtOAc:Hexane=1:3); mp: 161-162 ℃; UV (MeOH)：λ_{max} nm (logε) = 263 (3.91), 212(4.02); IR (KBr) cm^{-1}: 2923 (C-H), 1656 (C=O), 1618, 1600 (C=C), 1205 (C-C), 1170 (C-O), 1110 (C-Cl); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 1.62 (s, 3H, -CHC($C\underline{H}_3$)$_2$), 1.66 (s, 3H, -CHC($C\underline{H}_3$)$_2$), 3.40 (s, 1H, 1 of C6-C$\underline{H}_2$), 3.42 (s, 1H, 1 of C6-C$\underline{H}_2$), 3.88 (s, 3H, -ArOC$\underline{H}_3$), 5.13 (t, *J*=1.5 Hz, 1H, -C$\underline{H}$C(CH_3)$_2$), 6.40 (s, 1H, H-8), 6.51 (s, 1H, H-3), 7.38-7.62 (m, 4H, H-2', 3', 5', 6'), 12.72 (s, 1H, -C5-O$\underline{H}$); ^{13}C-NMR (75 MHz, $CDCl_3$-d_1): δ (ppm) = 17.7, 21.8, 25.8, 56.2, 90.0, 95.7, 105.6, 108.7, 111.2, 122.6, 127.4, 131.1, 131.2, 132.0, 132.1, 132.3, 133,3, 161.2, 163.4, 163.6, 181.1; HRMS (EI) *m/z*: calcd for $[M]^+$, 370.0972 ($C_{21}H_{19}ClO_4^+$), found：370.0968.

18) 2-(4-Chlorophenyl)-5-hydroxy-7-methoxy-6-(3-methylbut-2-en-1-yl)-4H-chromen-4-one (6c):

Rf: 0.52 (EtOAc:Hexane=1:3); mp: 165-167 ℃; UV (MeOH): λ_{max} nm (logε) = 277 (4.02), 215 (4.04) ; IR (KBr) cm^{-1}: 2920 (C-H), 1652 (C=O), 1643, 1616 (C=C), 1201 (C-C), 1172 (C-O), 1093 (C-Cl); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1); δ (ppm) = 1.68 (s, 3H, -CHC($C\underline{H}_3$)$_2$), 1.80 (s, 3H, -CHC($C\underline{H}_3$)$_2$), 3.53 (s, 1H, 1 of C6-C$\underline{H}_2$), 3.34 (s, 1H, 1 of C6-C$\underline{H}_2$), 3.92 (s, 3H, -ArOC$\underline{H}_3$), 5.22 (t, *J*=1.5 Hz, 1H, -C$\underline{H}$C(CH_3)$_2$), 6.49 (s, 1H, H-8), 6.62 (s, 1H, H-3), 7.47 (d, *J*=8.7 Hz, 2H, H-2', 6'), 7.80 (d, *J*=8.7 Hz, 2H, H-3', 5'), 12.70 (s, 1H, -C5-O$\underline{H}$); ^{13}C-NMR (75 MHz , $CDCl_3$-d_1): δ (ppm) = 17.9, 21.6, 25.8, 29.8, 56.1, 90.0, 106.5, 113.9, 122.3, 127.8, 127.9, 129.7, 130.5, 132.2, 138.4, 159.0, 164.0, 183.0; HRMS (EI) *m/z*: calcd for $[M]^+$, 370.0972 ($C_{21}H_{19}ClO_4^+$); found：370.0965.

19) 5-Hydroxy-7-(oxiran-2-ylmethoxy)-2-phenyl-4H-chromen-4-one (7a):

Rf: 0.41 (EtOAc:Hexane=1:2); mp: 178-180 ℃; UV (MeOH): λ_{max} nm (logε) = 268 (3.80), 211 (3.85); IR (KBr) cm^{-1} : 3066 (C-H), 1666 (C=O), 1616 (C=C), 1172 (C-O); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 2.78 (dd, *J* = 4.8 Hz, 2.7 Hz, , 1H, 1 of CHOC$\underline{H}_2$), 2.95 (t, *J* = 4.5 Hz, , 1H, 1 of CHOC$\underline{H}_2$), 3.38-3.41 (m, 1H, C$\underline{H}$OCH_2), 4.00 (dd, *J* = 11.1 Hz, 5.7 Hz, 1H, 1 of ArOC$\underline{H}_2$), 4.33 (dd, *J* = 11.1 Hz, 2.7 Hz, 1H, 1 of ArOC$\underline{H}_2$), 6.39 (d, *J* = 2.1 Hz, 1H, H-6), 6.54 (d, *J* = 2.1 Hz, 1H, H-8), 6.68 (s, 1H, H-3), 7.50-7.57 (m, 3H, H-3', 4', 5'), 7.87-7.90 (m, 2H, H-2', 6'), 12.73 (s, 1H, -C5-O$\underline{H}$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) = 43.2, 48.8, 69.2, 93.0, 98.1, 104.7, 105.0, 126.0, 128.7, 130.3, 131.6, 157.0, 160.9, 163.3, 163.9, 181.7; HRMS (EI) *m/z*: calcd for $[M]^+$, 310.0841 ($C_{18}H_{14}O_5^+$), found: 310.0845.

20) 2-(2-Chlorophenyl)-5-hydroxy-7-(oxiran-2-ylmethoxy)-4H-chromen-4-one (7b):

Rf: 0.43 (EtOAc:Hexane=1:2); mp: 187-189 ℃; UV (MeOH): λ_{max} nm (logε) = 305 (3.57), 260 (3940), 211 (4.01); IR (KBr) cm^{-1} : 3600~3200 (O-H), 2927 (C-H), 1672 (C=O), 1616 (C=C), 1178 (C-O), 1035 (C-Cl); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 2.77 (dd, *J* = 4.8 Hz, 2.7 Hz, , 1H, 1 of CHOC$\underline{H}_2$), 2.93 (t, *J* = 4.5 Hz, , 1H, 1 of CHOC$\underline{H}_2$), 3.36-3.39 (m, 1H, C$\underline{H}$OCH_2), 3.98 (dd, *J* = 11.1 Hz, 5.7 Hz, 1H, 1 of ArOC$\underline{H}_2$), 4.31 (dd, *J* = 11.1 Hz, 2.7 Hz, 1H, 1 of ArOC$\underline{H}_2$), 6.40 (d, *J* = 2.1 Hz, 1H, H-6), 6.47 (d, *J* = 2.1 Hz, 1H, H-8), 6.53 (s, 1H, H-3), 7.38-7.62 (m, 4H, H-2', 3', 5', 6'), 12.63 (s, 1H, -C5-O$\underline{H}$); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) = 43.5, 49.1, 69.66, 93.4, 98.7, 105.0, 110,8, 127.7, 130.4, 130.8, 131.2, 131.6, 132.7, 157.7, 161.4, 163.1, 164.5, 181.6; HRMS (EI) *m/z*: calcd for $[M]^+$, 344.0452 ($C_{18}H_{13}ClO_5^+$), found: 344.0455.

21) 2-(4-Chlorophenyl)-5-hydroxy-7-(oxiran-2-ylmethoxy)-4H-chromen-4-one (7c):

Rf: 0.45 (EtOAc:Hexane=1:2); mp: 208-210 ℃; UV (MeOH): λ_{max} nm (logε) = 270 (4.02), 213(4.04); IR (KBr) cm^{-1}: 3340 (O-H), 1656 (C=O), 1616 (C=C), 1164 (C-O), 1095 (C-Cl); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 2.78 (dd, *J* = 4.8 Hz, 2.7 Hz, , 1H, 1 of CHOC$\underline{H}_2$), 2.95 (t, *J* = 4.5 Hz, , 1H, 1 of CHOC$\underline{H}_2$), 3.38-3.41 (m, 1H, C$\underline{H}$OCH_2), 3.99 (dd, *J* = 11.1 Hz, 5.7 Hz, 1H, 1 of ArOC$\underline{H}_2$), 4.33 (dd, *J* = 11.1 Hz, 3.0 Hz, 1H, 1 of ArOC$\underline{H}_2$), 6.39 (d, *J* = 2.4 Hz, 1H, H-6), 6.53 (d, *J* = 2.4 Hz, 1H, H-8), 6.64 (s, 1H, H-3), 7.49 (d, *J* = 8.7 Hz, 2H, H-2', 6'), 7.81 (d, *J* = 8.7 Hz, 2H, H-3', 5'), 12.66 (s, 1H, -C5-O$\underline{H}$); ^{13}C-NMR (75 MHz, acetone- d_6): δ (ppm) = 44.5, 50.3, 71.0, 94.4, 99.7, 106.7, 106.9, 129.2, 130.4, 131.3, 138.7, 159.0, 163.5, 164.1, 166.1, 183.5; HRMS (EI) *m/z*: calcd for $[M]^+$, 344.0452 ($C_{18}H_{13}ClO_5^+$), found: 344.0454.

22) 3,4-Dihydro-7-hydroxy-5-methoxy-4-phenyl-2H-1-chromen-2-one (9):

Rf: 0.41 (EtOAc:Hexane=1:3); mp: 162-163 ℃; IR (KBr) cm^{-1}: 3600~3200 (broad, O-H), 3015, 2923 (C-H), 1741 (C=O), 1622 (C=C) ; ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 2.82 (dd, *J* = 17.1, 3.3 Hz, 1H), 3.27 (dd, *J* = 17.1, 10.8 Hz, 1H), 3.79 (s, 3H, -C5-OC$\underline{H}_3$), 5.62 (dd, *J* = 10.8, 3.3 Hz, 1H), 6.09 (d, *J* = 2.1 Hz, 1H, H-6), 6.14 (d, *J* = 2.1 Hz, 1H, H-8), 7.38-7.46 (m, 3H, H-3', 4', 5'), 7.18-7.27 7.54 (dd, 2H, *J* = 7.2, 1.5 Hz, H-2', 6'), 12.08 (s, 1H, -C7-O$\underline{H}$); HRMS (EI) *m/z*: calcd for $[M]^+$, 270.1916 ($C_{15}H_{12}O_4^+$); found: 270.1919.

23) 8-Phenyl-7,8-dihydropyranoflavanone (10):

Rf: 0.56 (EtOAc:Hexane=1:3); mp: 171-173 ℃; IR (KBr) cm^{-1}: 3105, 3012, 2955 (C-H), 1645 (C=O), 1623 (C=C) ; ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 2.07 (dd, *J* = 7.2, 2.1 Hz, 1H), 2.85-2.97 (m, 1H), 3.22 (dd, *J* = 7.2, 2.4 Hz, 1H), 4.59 (dd, *J* = 7.2, 2.1 Hz, 2H, H-2,8), 7.01-7.37 (m, 10H, Ar-H), 11.8 (s, 1H, -C5-O$\underline{H}$); MS (EI) 70 ev *m/z*: calcd for $[M]^+$, 386.41 ($C_{15}H_{12}O_4^+$); found , 386 (100%).

24) 2,3-Dihydro-5,7-dihydroxy-2-phenyl-4H-1-chromen-4-one (11):

Rf: 0.41 (EtOAc:Hexane=1:3); mp: 184-185 ℃; IR (KBr) cm^{-1}: 3600~3200 (broad, O-H), 3015, 2923 (C-H), 1741 (C=O), 1622 (C=C) ; ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 2.80 (dd, *J* = 15.9, 1.8 Hz, 1H, H-3), 3.19 (dd, *J* = 15.9, 7.2 Hz, 1H, H-3), 4.43 (dd, *J* = 7.2, 1.8 Hz, 1H, H-2), 6.02 (d, *J* = 2.1 Hz, 1H, H-6), 6.16 (d, *J* = 2.1 Hz, 1H, H-8), 7.05-7.08 (m, 2H, H-2', 6'), 7.18-7.27 (m, 3H, H-3', 4', 5'), 9.54 (s, 1H, -C7-O<u>H</u>); 9.72 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) = 30.7, 38.1, 96.8, 100.2, 105.3, 127.9, 128.1, 129.8, 143.9, 155.1, 156.7, 159.5, 168.3; HRMS (EI) *m/z*: calcd for $[M]^+$, 256.0737 ($C_{15}H_{12}O_4^+$). We found: 256.0739.

25) 5-O-(3-Methylbut-2-en-1-yl)-7-methoxy-2-phenyl-4H-chromen-4-one (12):

Rf: 0.57 (EtOAc:Hexane=1:3); mp: 188-190 ℃; IR (KBr) cm^{-1}: 3066, 2920 (C-H), 1645 (C=O), 1606 (C=C) ; ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 1.73 (s, 3H, -CHC(C<u>H</u>$_3$)$_2$), 1.89 (s, 3H, -CHC(C<u>H</u>$_3$)$_2$), 3.87 (s, 1H, 1 of C6-C<u>H</u>$_2$), 3.91 (s, 1H, 1 of C6-C<u>H</u>$_2$), 5.62 (t, *J*=2.4 Hz, 1H, -C<u>H</u>C(CH$_3$)$_2$), 6.36 (d, *J* =2.1 Hz,1H, H-6) 6.42 (d, *J* =2.1 Hz,1H, H-8), 6.66 (s, 1H, H-3), 7.55-7.59 (m, 3H, H-3', 4', 5'), 7.91-7.95 (m, 2H, H-2', 6'); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) = 17.8, 25.1, 55.7, 65.8, 93.4, 97.6, 108.2, 108.8, 119.7, 125.8, 128.8, 131.0, 131.2, 136.7, 159.2, 159.4, 159.5, 163.3, 175.5; HRMS (EI) *m/z*: calcd for $[M]^+$, 336.1362 ($C_{21}H_{20}O_4^+$), found：336.1359.

26) 5-Hydroxy-7-(3-methylglycidyloxy)-flavone (13):

Rf: 0.47 (EtOAc:Hexane = 1:2); mp: 183-185 ℃; UV (MeOH): λ_{max} nm (logε) = 265 (3.89), 215 (3.83); IR (KBr) cm^{-1}：3600~3200 (broad, O-H), 3012, 2906 (C-H), 1657 (C=O), 1612 (C=C); ^{1}H-NMR (300 MHz, $CDCl_3$-d_1): δ (ppm) = 3.35-3.47 (m, 2H), 3.95-4.01 (m, 2H), 3.98 (s, 3H, OC<u>H</u>$_3$), 5.22 (d, *J* = 4.8 Hz, 1H), 6.37 (d, *J* = 2.1 Hz, 1H, H-6), 6.81 (d, *J* = 2.1 Hz, 1H, H-8), 7.01 (s, 1H, H-3), 7.54-7.62 (m, 3H, H-3', 4', 5'), 8.07-8.10 (m, 2H, H-2', 6'), 12.77 (s, 1H, -C5-O<u>H</u>); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) = 43.2, 48.8, 69.2, 93.0, 98.1, 104.7, 105.0, 126.0, 128.7, 130.3, 131.6, 157.0, 160.9, 163.3, 163.9, 181.7; HRMS (EI) *m/z*: calcd for $[M]^+$, 342.2239 ($C_{19}H_{18}O_6^+$), found: 342.2241.

C. In Vitro Evaluations

Virus- Two influenza virus strains, oseltamivir (Tamiflu)-resistant 2009 pandemic influenza A (H1N1) virus, which detected the H275Y mutation (N1 numbering) in neuraminidase and Influenza A/New York/469/2004-like flu virus, were provided by Centers for Disease Control (CDC, Taiwan) and adapted for evaluating flavonoids of in vitro anti-viral activities.

Cytotoxicity in MDCK cells- Cell viability was determined by the standard manipulation of MTT assay [24]. Madin Darby canine kidney (MDCK, American Type Culture Collection, catalog # CCL-34) cells were incubated 1 x 10^5 cells/well in 96-well plates for 24 h at 37 ℃ in a 75% humidity of 5% CO_2 atmosphere. The plates containing a confluent cell monolayer in triplicate were replaced with media containing serially 2-fold diluted compounds (1 to 400 μM of the respective flavonoids and ribavirin), and blank medium was used as the control. After 48 h of incubation, the medium was removed and 5 μL of MTT solution (3-(4,5-dimethylthiozol-2-yl)-3,5-dipheryl tetrazolium bromide 5 mg/ml in phosphate buffered saline) was added to each well and incubated at 37 ℃ for 4 h. After the removal of supernatant, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals. After 15 min, the plates were homogenized on a microplate shaker. Absorbance was measured at 540 nm with subtraction of the background measurement at 655 nm in a microplate reader. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis.

Assay of cytopathic effect (CPE)- The anti-viral activity of flavonoids was measured by the cytopathic effect (CPE) assay and using ribavirin as a positive control. The CPE inhibition assays used in this study were performed as described previously [25]. In brief, virus at 100 $TCID_{50}$ (tissue culture infectious dose) were inoculated onto near confluent MDCK cell monolayers (1 x10^5 cells/well) for 1 h. After being incubated at 37 ℃ for 2 h, the virus solution was removed, and 100 μL sequential 2-fold serial dilutions of the respective flavonoids and reference compound ribavirin were added to each well of the 96-well culture plates, using the maximal non-cytotoxic concentration (MNCC, i.e. 90% viable cells) as the highest concentration. An infection control without flavonoids was also included. The plates were incubated at 37 ℃ in a 75% humidity of 5% CO_2 atmosphere for 24 h, and then the CPE was observed. The virus-induced CPE was scored as follows: scores: 0 = 0% CPE, 1 = 0–25% CPE, 2 = 25–50% CPE, 3 = 50–75% CPE, and 4 = 75–100% CPE. The reduction in virus multiplication was calculated as a percentage of the virus control (% virus control = CPEexp/CPEvirus control x 100). The IC_{50} of the CPE with respect to virus control was estimated using the Reed–Muench method and was expressed in μM. The selectivity index (SI) was calculated from the ratio CC_{50}/IC_{50}.

D. Statistical Analysis

Statistical calculations were carried out with Microsoft Excel 2007 version. Results are expressed as the means ± SD of six independent experiments.

Ⅴ. CONCLUSIONS

In this study, we had conducted practically standard manipulations to synthesize a series of novel flavonoids relevant to baicalein/oroxyllin-A, derived from Scutellaria baicalensis GEORGI. All the synthesized flavonoids in this study were screening anti-influenza activity against influenza virus (H1N1-Tamiflu resistant and H3N2)-infected in MDCK cell line. Our results showed that most of the synthetic products possessed significant activity compared to ribavirin (a positive control) against H1N1-Tamiflu resistant and/or H3N2 viruses. Eventually, the findings of this study provide somewhat important chemical structural features of flavonoids relating their ability after SAR analysis to control the replication of influenza virus. Those results would provide basis to facilitate the design and development of chemical compounds with higher potency to serve as potential NA inhibitors for influenza treatment.

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REFERENCES

[1] W. H. Huang, P. Y. Chien, C. H. Yang, and A. R. Lee, "Novel synthesis of flavonoids of *Scutellaria baicalensis* Georgi." *Chem. Pharm. Bull.*, vol 51(3), pp. 339–340, Mar. 2003.

[2] W.H. Huang, A.R. Lee, P.U. Chien, and T.C. Chou, “Synthesis of baicalein derivatives as potential anti-aggregatory and anti-inflammatory agents.” *J. Pharm. Pharmacol.*, vol. 57, pp. 219–225, 2005.

[3] W. H. Huang, A.R. Lee, and C.H. Yang, “Antioxidative and anti-inflammatory activities of polyhydroxyflavonoids of *Scutellaria baicalensis* GEORGI.” *Biosci. Biotechnol. Biochem,* vol. 10, pp. 2371–2380, Oct. 2006.

[4] P. Meindl, G. Bodo, P. Palese, J. L. Schulman, and H. Tuppy, “Inhibition of neuraminidase activity by derivatives of 2-deoxy-2, 3-dehydro-N-acetylneuraminic acid.” *Virology*, vol. 58, pp. 457–463, Apr. 1974.

[5] A. K. Chong, M. S. Pegg, N. R. Taylor, and M. von Itzstein, “Evidence for a sialosyl cation transition-state complex in the reaction of sialidase from influenza virus.” *Eur. J. Biochem.*, vol. 207(1), pp. 335–343, Jul. 1992.

[6] N. R. Taylor and M. von Itzstein, “Molecular modeling studies on ligand binding to sialidase from influenza virus and the mechanism of catalysis.” *J. Med. Chem.*, vol. 37(5), pp. 616–624, Mar. 1994.

[7] E. D. Clercq, “Antiviral agents active against influenza A virus.” *Nature Rev. Drug Discov.*, vol. 5, pp. 1015–1025, Dec. 2006.

[8] J. Zhang and W. F. Xu, “Recent advances in anti-influenza agents with neuraminidase as target.” *Mini Rev. Med. Chem.*, vol. 6, pp. 429–448, Apr. 2006.

[9] I.M. Lagoja and E. D. Clercq, “Anti-influenza virus agents: Synthesis and mode of action.” *Med. Res. Rev.*, vol. 28 (1), pp. 1–38. Jan. 2008.

[10] M. von Itzstein “The war against influenza: Discovery and development of sialidase inhibitors.” *Nature Rev. Drug Discov.*, vol. 6, pp. 967–974, Dec. 2007.

[11] J. Beigel and M. Bray, “Current and future antiviral therapy of severe seasonal and avian influenza.” *Antiviral Res.*, vol. 78 (1), pp. 91–102, Apr. 2008.

[12] I. Fuyuno, "Tamiflu side effects come under scrutiny." *Nature*, vol. 446 (7134), 358–359, Mar. 2007.

[13] P. J. Collins, L. F. Haire, Y. P. Lin, J. Liu, R. J. Russell, P. A. Walker, J. J. Skehel, S. R. Martin, A. J. Hay and S. J. Gamblin, “Crystal structures of oseltamivir-resistant influenza virus neuraminidase mutants.” *Nature*, vol. 453, pp. 1258–1262, Jun. 2008.

[14] A. Moscona, “Global transmission of oseltamivir-resistant influenza.” *N. Engl. J. Med.*, vol. 360 (10), pp. 953–956, Mar. 2009.

[15] J. Gooskens, M. Jonges, E. C. J. Claas, A. Meijer, P. J. van den Broek, and A. C. M. Kroes, “Morbidity and mortality associated with nosocomial transmission of oseltamivir-resistant influenza A (H1N1) virus.” *JAMA*, vo. 301, pp. 1042–1046, Mar. 2009.

[16] T. Nagai, Y. Miyaichi, T. Tomimori, Y. Suzuki, and H. Yamada, “Inhibition of influenza virus sialidase and anti-influenza virus activity by plant flavonoids.” *Chem. Pharm. Bull.*, vol. 38(5), pp. 1329–1332, Mar. 1990.

[17] T. Nagai, Y. Miyaichi, T. Tomimori, Y. Suzuki, and H. Yamada, “In vivo anti-influenza virus activity of plant flavonoids possessing inhibitory activity for influenza virus sialidase.” *Antiviral Res.*, vol. 19, pp. 207–217, Sept. 1992.

[18] T. Nagai, R. Moriguchi, Y. Suzuki, T. Tomimori, and H. Yamada, “Mode of action of the anti-influenza virus activity of plant flavonoid, 5,7,4'-trihydroxy-8-methoxyflavone, from the roots of Scutellaria baicalensis.” *Antiviral Res*, vol. 26, pp. 11–25, Ian. 1995.

[19] R. J. Nijveldt, E. van Nood, D. EC. van Hoorn, P. G. Boelens, K. van Norren, and P. AM. van Leeuwen, “Flavonoids：a review of probable mechanisms of action and potential applications.” *Am. J. Clin. Nutr.*, vol. 74, 418–425, Oct. 2001.

[20] A. L. Liu, H. D. Wang, S. M. Y. Lee, Y. T Wang., and G. H. Du, “Structure–activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities.” *Bioorg. Med. Chem.*, vol. 16, pp. 7141–7147, Aug. 2008.

[21] H. J. Jeong, Y. B. Ryu, S. J. Park, J. H. Kim, H. J. Kwon, J. H. Kim, K. H. Park, M. C. Rho, and W.S. Lee, “Neuraminidase inhibitory activities of flavonols isolated from *Rhodiola rosea* roots and their in vitro anti-influenza viral activities.” *Bioorg. Med. Chem.*, vol. 17, pp. 6816–6823, Oct. 2009.

[22] Y. B. Ryu, M. J. Curtis-Long, J. W. Lee, H. W. Ryu, J. Y. Kim, W. S. Lee, and K. H. Park, “Structural characteristics of flavanones and flavones from *Cudrania tricuspidata* for neuraminidase inhibition.” *Bioorg. Med. Chem. Lett.* vol. 19, pp. 4912–4915, Sept. 2009.

[23] G.Xu, J. Dou, L. Zhang, Q. Guo, and C. Zhou, “Inhibitory effects of baicalein on the influenza virus in vivo is determined by baicalin in the serum.” *Biol. Pharm. Bull.* Vol. 33, pp. 238–243, Feb. 2010.

[24] L. Chen, J. Dou, Z. Su, H. Zhou, H. Wang, W. Zhou, Q. Guo, and C. Zhou, “Synergistic activity of baicalein with ribavirin against influenza A (H1N1) virus infections in cell culture and in mice.” *Antiviral Res., vol.* 91, pp. 314–320, Jul. 2011.

[25] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays". *J. Immunolog. Method.*, vol. **65** (1–2), pp. 55–63, Dec. 1983.

Wen-Hsin Huang, Ph.D. The corresponding author is currently an associate professor, School/Institute of Pharmacy, National Defense Medical Center (NDMC) at Taipei, Taiwan. E-mail: wenhsin@ndmctsgh.edu.tw; date of birth: Oct. 31, 1962. He earned a Ph. D. degree at 2000 in the College of Pharmacy from the University of Texas at Austin, Austin city, Texas, USA.

Dr. Huang’s researches focus on active components of the synthetic chemistry derived from the Traditional Chinese Medicines on drug design and development of novel anti-angiogenic anticancer agents, bioreductive anticancer agents, anti-diabetic agents, anti-viral agents, anti-inflammatory agents, antioxidants and immunosuppresants and further screening of bioassay.